Glutamine Starvation of Murine Leukaemia Virus-infected Cells Inhibits the Readthrough of the gag–pol Genes and Proteolytic Processing of the gag Polyprotein

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SUMMARY

The production of Moloney murine leukaemia virus from chronically infected cells was inhibited after starvation of glutamine. While the rate of synthesis of the precursor of the core proteins, Pr65gag, was not affected in the starved cells, its proteolytic processing was blocked. Pulse–chase experiments indicated that glutamine was required during the synthesis of Pr65gag to facilitate its subsequent processing. In addition, the synthesis of Pr200gag–pol, the precursor of the protease, reverse transcriptase and endonuclease, was inhibited in the glutamine-starved cells. Starvation for other essential amino acids such as tyrosine and isoleucine affected neither the synthesis nor the processing of the virus proteins. These results suggest that the readthrough mechanism which enables synthesis of the Pr200gag–pol polyprotein is modulated in the chronically infected cells by glutamine levels. Since the viral protease is part of the pol gene, its synthesis may be inhibited in the glutamine-starved cells and Pr65gag is therefore not processed.

INTRODUCTION

The genome of murine leukaemia viruses (MuLV) contains three genes, termed gag, pol and env, whose expression is regulated at both the transcription and translation levels. The major gag gene product is a polyprotein of 65 000 mol. wt. (Pr65gag) which is translated from the 35S viral RNA and processed into the four virus structural proteins p15, p12, p30 and p10 (Kerr et al., 1976; Jamjoom et al., 1977). The protease responsible for this cleavage appears to be encoded within the 5′ portion of the pol gene of MuLV (Crawford & Goff, 1985; Levin et al., 1984; Yoshinaka et al., 1985). The pol gene product is a larger polyprotein, Pr200gag–pol, which appears also to be translated from the 35S viral RNA and is processed into the mature reverse transcriptase (p85) (Jamjoom et al., 1977; Panet et al., 1975; Kopchick et al., 1978).

The amount of Pr200gag–pol produced in MuLV chronically infected cells is only 5 to 10% that of Pr65gag. Translation of the 35S viral RNA in vitro in crude reticulocyte extracts also resulted in a ratio of about 1:20 between these two polyprotein products (Murphy et al., 1978; Philipson et al., 1978). Addition of yeast amber suppressor tRNA and to a lesser extent ochre, dramatically changed this ratio to increase the amount of Pr200gag–pol (Philipson et al., 1978). Based on these observations, it has been proposed that the MuLV pol gene is translated by readthrough of a termination codon present between the gag and pol genes. In fact, sequence analysis of the Moloney MuLV (M-MuLV) genome has revealed a UAG amber codon in the correct reading frame between these two genes (Shinnick et al., 1981). Analysis of amino acid residues at the NH₂ terminus of the M-MuLV protease indicated that this amber codon is read as glutamine in the Pr200gag–pol (Yoshinaka et al., 1985).

The low efficiency of the amber codon readthrough in the infected cell is possibly due to competition of the putative glutamine suppressor with translation release factors. To elucidate the nature of this suppression mechanism and its control in vitro, we have analysed the effect of glutamine levels on the synthesis and processing of MuLV proteins in chronically infected cells.
METHODS

Cell cultures. Mouse NIH/3T3 cells chronically infected with M-MuLV (clone 1; Fan & Paskind, 1974) were grown in RPMI 1640 medium containing 10% calf serum. For amino acid deprivation, 10^6 cells were plated in 90 mm dishes for 24 h; the monolayers were washed with Eagle's MEM (MEM-E) without amino acids and then fed with MEM-E medium lacking glutamine (Gln) where specified and containing 5% dialysed foetal calf serum. After 24 h of amino acid starvation, the cells were pulse-labelled with [35S]methionine ([35S]Met), 15 μCi/ml (Amersham) in MEM-E medium without Met or Gln. In the pulse-chase experiments, after 90 min of incubation with [35S]Met, the medium was replaced with MEM-E containing 10% calf serum.

Immuno precipitation. Immuno precipitation of M-MuLV proteins was carried out as described previously (Gloger et al., 1985; Witte & Baltimore, 1978). In brief, cells were extracted in 10 mM-sodium phosphate pH 7.5, 100 mM-NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate and clarified by centrifugation. Since the Gln-deprived cultures incorporated about 50% of the [35S]Met incorporated by the control cells, equal amounts of acid-insoluble radioactivity were incubated with 5 μl of rabbit or goat immune sera. After overnight incubation at 4°C, a suspension of Staphylococcus aureus (50 μl; 10^8, v/v) was added and the complexes were washed with the extraction buffer. Proteins were resolved by electrophoresis in SDS-12.5% polyacrylamide gels. Protein markers for determination of molecular weights (Sigma) were always included in the gels and stained with Coomassie Brilliant Blue.

Antisera. Rabbit antisera against purified M-MuLV p30 and reverse transcriptase (Philipson et al., 1978) was a gift from U. Olshevsky, The Biological Institute, Nes-Ziona, Israel. Goat antisera against purified Rauscher MuLV p30 and reverse transcriptase were obtained through the Office of Logistics, NCI, Bethesda, Md., U.S.A. Antisera from these two sources gave identical results. Specificity of immunoprecipitation was checked by (i) using non-immune sera and (ii) competition with purified virions of M-MuLV.

Cell DNA synthesis. DNA synthesis was determined by measuring the incorporation of [3H]thymidine into TCA-insoluble material after 1 h pulse labelling of the cultures (Gloger et al., 1985).

RESULTS

In previous work we have investigated the effect of cell cycle arrest on the replication of MuLV in chronically infected cells (Gloger et al., 1985). In cells arrested at the G0/G1 phase by deprivation of Gln and isoleucine (Ile) (Yen & Pardee, 1978), virus production was blocked.

To determine whether the inhibition of virus production by Gln/Ile deprivation was related to cell arrest or to other possible effects of amino acid starvation, we grew the cells in the absence of one amino acid at a time and compared viral protein synthesis and cell DNA synthesis. Viral protein synthesis was determined after pulse-labelling the cells with [35S]Met and immunoprecipitation from crude extracts with anti-p30 sera (Fig. 1). Depletion of Gln alone or Gln plus Ile from the growth medium for 48 h resulted in 90% inhibition of cell proliferation, as indicated by the inhibition of [3H]thymidine incorporation into cellular DNA (Gloger et al., 1985). Under these conditions, Pr65</sub>ag was synthesized but its processing into p30 was blocked (compare Fig. 1, lanes 1, 2 and 3). On the other hand, depletion of Ile from the growth medium inhibited cell proliferation by 80% without affecting the processing of Pr65</sub>ag into p30 (Fig. 1, lane 5). Deprivation of tyrosine, another essential amino acid for these cells, had very little effect on either cell DNA synthesis (10% inhibition compared to non-starved cells), or the processing of Pr65</sub>ag (Fig. 1, lane 4). Note that a minor double band of high molecular weight protein (indicated by an arrow) was usually visible in samples from cells in which Pr65</sub>ag processing took place and was absent in the Gln-deprived cells (Fig. 1 and 2). As shown later (Fig. 5), this protein appears to represent Pr200</sub>ag–pol. The results presented above indicated to us that inhibition of the cell cycle by amino acid deprivation may not be the primary cause for the inhibition of Pr65</sub>ag processing.

To study further the mechanism of inhibition of Pr</sub>65</sub>ag cleavage, we analysed the period of Gln starvation required to block processing (Fig. 2a). Deprivation of Gln for 48 h or 24 h efficiently inhibited the processing (Fig. 2a, lanes 2 and 3). Shorter starvation periods, 6 h (lane 4) and 2 h (lane 5) before the pulse-labelling only partially inhibited Pr65</sub>ag processing. In parallel, we tested whether Gln may be added back to the starved cells before pulse-labelling to facilitate Pr65</sub>ag processing (Fig. 2b). Addition of Gln to starved cells either 2 h prior to the pulse-labelling (lane 1), or even during the 90 min labelling period (lane 2), indeed restored processing into p30. This would suggest that Gln is needed during synthesis of Pr65</sub>ag to enable its subsequent processing.
Readthrough of the MuLV gag-pol genes

Fig. 1. Deprivation of different amino acids and the effect on processing of Pr65gag. Cells were grown for 48 h in MEM-E without the specified amino acids and labelled for 90 min with [35S]Met in medium without Met and the amino acid used for starvation. Lane 1, minus Gln; lane 2, control cells in complete medium; lane 3, minus Gln and Ile; lane 4, minus Tyr; lane 5, minus Ile.

Fig. 2. The Gln starvation period needed to inhibit processing of Pr65gag. (a) Cells were deprived of Gln for different periods and labelled in parallel for 90 min with [35S]Met. Lane 1, control cells not starved; lane 2, 48 h starvation; lane 3, 24 h starvation; lane 4, 6 h starvation; lane 5, 2 h starvation. (b) Cells were deprived of Gln for 48 h and changed to complete medium 2 h before radiolabelling (lane 1) or during radiolabelling (lane 2). The cells were pulse-labelled with [35S]Met for 90 min.
Fig. 3. Effect of Gln on the processing of Pr65\textsuperscript{gag} which had been prelabelled in Gln-starved cells. Cells were starved of Gln for 24 h and pulse-labelled with \[^{35}\text{S}]\text{Met} in the absence of Gln for 90 min. The medium was changed and incubation (chase) was continued for 90 min and 8 h. Lane 1, control unstarved cells, pulse-labelled; lane 2, Gln-starved cells, pulse-labelled; lane 3, control unstarved cells chased for 90 min; lane 4, Gln-starved cells, chased for 90 min in the presence of Gln; lane 5, Gln-starved cells, chased for 90 min in the absence of Gln; lane 6, control unstarved cells, chased for 8 h; lane 7, Gln-starved cells, chased for 8 h in the presence of Gln; lane 8, Gln-starved cells, chased for 8 h in the absence of Gln.

Two possible explanations for the effect of Gln on the processing of Pr65\textsuperscript{gag} were considered: (i) an altered Pr65\textsuperscript{gag} was translated in Gln-starved cells, which was not recognized by the M-MuLV protease and (ii) the M-MuLV protease was not produced in the Gln-deprived cells.

To investigate these possibilities we added Gln during a chase period which followed pulse-labelling of the cells (Fig. 3). In the control non-starved cells, the pulse-labelled Pr65\textsuperscript{gag} was completely processed into p30 within 90 min of the chase (lanes 1 and 3). On the other hand, when starved cells were pulse-labelled in the absence of Gln (lane 2) and chased in the presence of Gln for 90 min or 8 h (lanes 4 and 7), slower processing into p30 was observed. It appeared that most Pr65\textsuperscript{gag} was degraded during the 8 h chase with Gln. When both the pulse-labelling and the chase were carried out in the absence of Gln, specific processing into p30 was, as expected, very poor (compare lanes 2, 5 and 8 in Fig. 3). It is interesting to note that in repeated experiments the stability of the pre-labelled Pr65\textsuperscript{gag} was significantly higher when the chase was conducted without Gln, as compared to cells where Gln was added during the chase. This would suggest that the factor needed for Pr65\textsuperscript{gag} processing may be synthesized during the chase in the presence of Gln to stimulate Pr65\textsuperscript{gag} cleavage.

Pr65\textsuperscript{gag} produced in the Gln-deprived cells appeared identical to the polyprotein made in control cells by two criteria: (i) migration in polyacrylamide gels (Fig. 3) and (ii) the corresponding cleavage products had the same migration on polyacrylamide gels when the Pr65\textsuperscript{gag} bands were excised from the gel and digested with V8 protease (data not shown).

Several of the conditional mutants of MuLV have been shown to be thermolabile for a function needed at a late step of virus replication. The lesions in these mutants affected both Pr65\textsuperscript{gag} processing and MuLV production (Stephenson et al., 1975; Witte & Baltimore, 1978). The thermolabile function appeared to be related to a proteolytic activity, as the addition of trypsin to the chronically infected cells grown at the non-permissive temperature resulted in processing of Pr65\textsuperscript{gag} and subsequent MuLV production (Traktman & Baltimore, 1982). To test whether exogenous protease would similarly overcome the effect of Gln deprivation, the cultures were pulse-labelled with \[^{35}\text{S}]\text{Met} and then treated with increasing amounts of trypsin (Fig. 4).
Fig. 4. Effect of trypsin on the processing of Pr65\textsuperscript{gag} in Gln-starved cells. Cells were deprived of Gln for 48 h, pulse-labelled with \textsuperscript{35}S\textsuperscript{Met} for 90 min, washed and treated with trypsin. Lane 1, control unstarved cells; lane 2, Gln-deprived cells; lane 3, Gln-deprived cells treated with trypsin (2 \mu g/ml); lane 4, Gln-deprived cells treated with trypsin (10 \mu g/ml). Treatment of cultures with pure trypsin (Sigma) in phosphate-buffered saline (5 ml) was for 15 min at 37 °C. The enzyme reaction was stopped by the addition of cold MEM-E containing 10% calf serum and 3 \mu g/ml phenylmethylsulphonyl fluoride. Trypsin-treated cell suspensions were centrifuged for 10 min at 2000 \textit{g} 4 °C, and the pellets were dissolved in immunoprecipitation extraction buffer.

There was no processing of Pr65\textsuperscript{gag} into p30 in the trypsin-treated cells. Some cleavage to an intermediate form of 52000 mol. wt. was catalysed by the trypsin (lanes 3 and 4, Fig. 4). This polypeptide might be similar to Pr55\textsuperscript{gag} (Ledbetter, 1979). It should be noted that M-MuLV was not released from the Gln-deprived cells after such exogenous trypsin treatment. Thus, Gln deprivation and the thermolabile function in the MuLV late gene mutants (Stephenson \textit{et al.}, 1975; Traktman & Baltimore, 1982) probably affect different stages of MuLV maturation.

The absence of the M-MuLV protease (Yoshinaka \textit{et al.}, 1985) in the Gln-deprived cells is likely to affect processing of Pr65\textsuperscript{gag}. Since this protease is coded as part of the \textit{pol} gene, we analysed the synthesis of Pr200\textsuperscript{gag-pol} in Gln-deprived cells. As expected, both anti-p30 and anti-reverse transcriptase sera immunoprecipitated the Pr200\textsuperscript{gag-pol} precursor from the control cell extracts (Fig. 5, lanes 1 and 3). This M-MuLV polyprotein usually appeared as a double band on the gels (Crawford & Goff, 1985). The ratio of the two bands varied in repeated experiments and in lane 1 the upper band predominated. The synthesis of Pr200\textsuperscript{gag-pol} was clearly suppressed in the Gln-starved cells (lanes 2 and 4). A protein of 160000 mol. wt. was immunoprecipitated with anti-reverse transcriptase serum (lane 3), but not with anti-p30 serum (lane 1). This protein, which probably represented a processing intermediate of the Pr200\textsuperscript{gag-pol} that lacks gag peptides (Crawford & Goff, 1985), was strongly inhibited in the Gln-deprived cells (lane 4). The specificity of the immunoprecipitation with anti-reverse transcriptase sera was demonstrated by displacement of the labelled double band Pr200\textsuperscript{gag-pol} and the protein of 160000 mol. wt. by excess M-MuLV virion proteins (results not shown).

**DISCUSSION**

We demonstrate in this work that the level of Gln in MuLV chronically infected cells regulated both the synthesis of Pr200\textsuperscript{gag-pol} and the processing of Pr65\textsuperscript{gag}. These effects are specific for Gln, as starvation for other essential amino acids did not affect the virus. It has
recently been reported that the protease responsible for processing of Pr65\textsuperscript{gag} is coded at the 5' end of the MuLV \textit{pol} gene (Crawford & Goff, 1985; Yoshinaka \textit{et al.}, 1985). Moreover, a glutamine residue was found at the fifth position from the NH\textsubscript{2} end of MuLV protease; this position is coded by an amber codon at the junction of \textit{gag} and \textit{pol} genes (Shinnick \textit{et al.}, 1981; Yoshinaka \textit{et al.}, 1985). In view of these results, we suggest that the level of cellular Gln may affect readthrough of the amber codon. Reduction of the readthrough frequency is expected to affect the synthesis of the protease precursor (Pr200\textsuperscript{gag-pol}) and thus the processing of Pr65\textsuperscript{gag}. During the work we also considered the possibility that in the Gin-starved cells Pr65\textsuperscript{gag} is sequestered into a different cell compartment, such that it is inaccessible to the viral protease. Since Pr65\textsuperscript{gag} is mainly associated with the cytoskeleton fraction of the cell, we have purified cytoskeletons from control and Gin-starved cells and compared their Pr65\textsuperscript{gag} content; no significant differences in the amounts of Pr65\textsuperscript{gag} were found.

Under conditions of Gin starvation, total cell protein synthesis is reduced by only 50\% and the rates of Pr65\textsuperscript{gag} and Pr80\textsuperscript{env} synthesis are not affected at all. This would suggest that the level of glutamine tRNAs in the starved cells is still sufficient to recognize the 24 normal Gln codons present in the \textit{gag} gene. Both Gin codons CAA and CAG are used but the later codon predominates.

Translation studies of M-MuLV 35S RNA \textit{in vitro} have indicated that exogenous yeast suppressor tRNAs increased readthrough of the Pr200\textsuperscript{gag-pol} (Philipson \textit{et al.}, 1978). It was not clear, however, whether in the chronically infected mouse cells an amber suppressor tRNA\textsuperscript{Gln} or one of the normal tRNA\textsuperscript{Gln} species is involved in reading the amber codon as Gin. If tRNA\textsuperscript{Gln} is involved in this readthrough, it is conceivable that cell starvation of Gin might reduce the level of tRNA\textsuperscript{Gln} aminoacylation such that reading of the amber codon between the \textit{gag} and \textit{pol} genes is preferentially inhibited.
Several RNA bacteriophages (Engelberg-Kulka et al., 1979) and Sindbis virus (Strauss et al., 1983) use stop codon readthrough as part of their translation strategies. The observation that starvation for a specific amino acid inhibited the synthesis of MuLV readthrough protein in vivo might be applied to screen other virus systems for proteins which are synthesized by similar mechanisms and to inhibit replication of these viruses.

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